Lentiviral shRNA expression Cloning Kit User Manual

for making shRNA expression lentivectors

Cat#	Product Name	Amount	Application
LTSH-GB	pEco-Lenti-H1-shRNA-(**GFP-Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with GFP-Blasticidin selection marker
LTSH-GP	pEco-Lenti-H1-shRNA-(**GFP-Puro**) cloning kit	1 kit	Make shRNA expression lentivector with GFP-Puromycin selection marker
LTSH-RB	pEco-Lenti-H1-shRNA-(**RFP-Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with RFP-Blasticidin selection marker
LTSH-RP	pEco-Lenti-H1-shRNA-(**RFP-puro**) cloning kit	1 kit	Make shRNA expression lentivector with RFP-Puromycin selection marker
LTSH-Puro	pEco-Lenti-H1-shRNA-(**puro**) cloning kit	1 kit	Make shRNA expression lentivector with Puromycin selection marker
LTSH-Bsd	pEco-Lenti-H1-shRNA-(**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with Blasticidin selection marker

Each Kit Contents	Amount
One of the following pre-cut, linear vector (dependent upon the Catalog #)**:** **pEco-Lenti-H1-(GFP-Bsd) linear vector;** **or: pEco-Lenti-H1-(GFP-Puro) linear vector;** **or: pEco-Lenti-H1-(RFP-Bsd) linear vector;** **or: pEco-Lenti-H1-(RFP-Puro) linear vector;** **or: pEco-Lenti-H1-(Puromycin) linear vector;** **or:pEco-Lenti-H1-(Blasticidin) linear vector;**	10ul (10rxn)
10x shRNA oligo annealing solution	50ul
5x ligation buffer	50ul

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T4 ligase enzyme	10ul (10rxn)
Cloning control insert: annealed Luc-shRNA duplex [1x]: 10ul (10rxn) 5'- AGCGatgaaacgatatgggctgaatacCGAGgtattcagcccatatcgtttca tactttgctatacccgacttatgGCTCcataagtcgggtatagcaaagtAAAA	
Sequencing primer: 5'- ggatccaatatttgcatgtcgctatg	1 tube (10ul x 25ng/ul) (10 rxn)

Note: Chemical competent cells are required for the cloning, but not included in this kit. You can use any common chemical competent cells, like DH5a, NovaBlue or others.

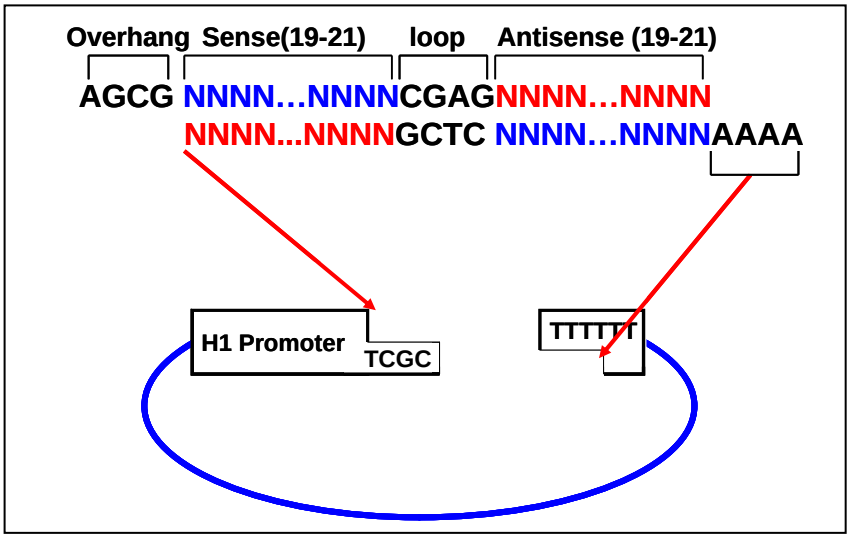
Storage:

shRNA Cloning Kit is shipped on dry ice. Each kit contains sufficient amounts for **10 shRNA cloning reactions**. The kit should be stored at -20°C. Products stable for 6 months.

Quick protocol (for experienced users):

1. Design two DNA oligonucleotides encoding shRNA sequence,
2. Anneal the two oligo to generate a duplex;
3. Clone the duplex into provided linear **pEco-shRNA** vector by T4 ligation reaction;
4. transform into competent cells and grow in LB/ ampicillin plate;
5. confirm positive clone by sequencing;
6. knockdown analysis after transfection of shRNA plasmids into mammalian cells;
7. Produce shRNA lentivirus and transduce into desired cells for knockdown analysis or generate shRNA stable cell lines.

Cloning Scheme



```
                                H1 promoter
1       GGATCCAATA TTTGCATGTC GCTATGTGTT CTGGGAAATC ACCATAAACG
        CCTAGGTTAT AAACGTACAG CGATACACAA GACCCTTTAG TGGTATTTGC
                     TRBS                              TRBS
              ~~~~~~~~~~~~~~~~~~~~~~~~          ~~~~~~~~~~~~~~~~~~~
51      TGAAATCCCT ATCAGTGATA GAGACTTATA AGTTCCCTAT CAGTGATAGA
        ACTTTAGGGA TAGTCACTAT CTCTGAATAT TCAAGGGATA GTCACTATCT

                Transcription start
        ~~
101     GA        TTTTTTGGCCGGCC ACCGGTTAGT AATGATCGAC AATCAACCTC
        CTTCGC          AACCGGCCGG TGGCCAATCA TTACTAGCTG TTAGTTGGAG

TRBS: Tetracycline Repressor Binding Site.
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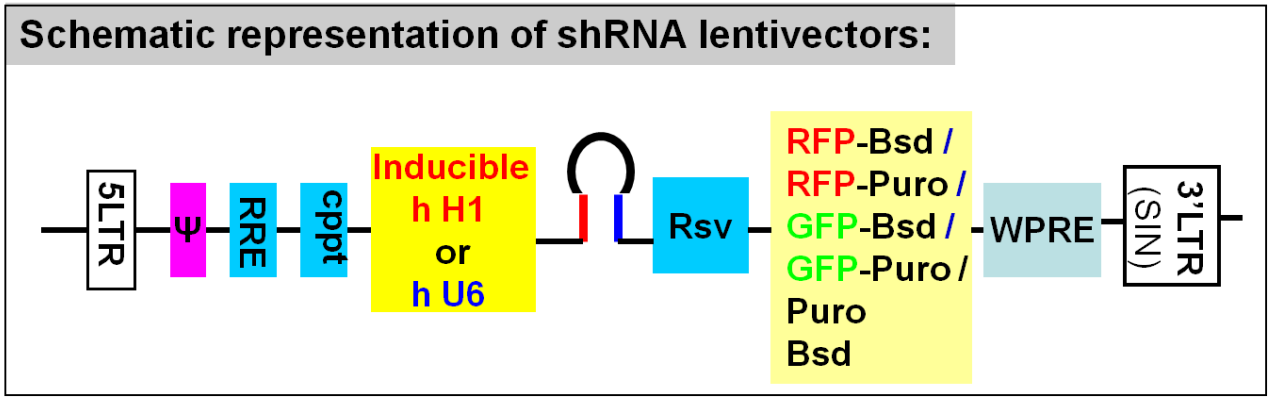
Vector Schematic maps



Introduction:

RNA interference (RNAi) technology is a tool for loss-of-function (knockdown / silencing) studies in mammalian cells without making knock-out germline cells. Originally, double-strand short RNAs were found *in vivo*, inhibiting gene expression. The mechanism is a series of enzymatic reactions mediated by short RNAs that are complementary in sequence to the silenced targets, leading to mRNA degradation or translational repression. RNAi knockdown can be introduced by synthetic short double-strand RNA (siRNA) or vector expressed stem-hairpin RNA (shRNA) which is further processed by Dicer enzyme to produce double-strand short RNAs. Another category of RNAi found *in vivo* is micro-RNA (miRNA) which has similar knockdown mechanism. Native or artificial miRNA can be processed from pre-micro RNA that is expressed via vector. Chemically synthesized double stranded RNA (siRNA) is

only for transient silencing effect. In contrast, vector expressed RNAi can provide a long term effect by stable selection.

Vector expressed RNAi for gene silencing provides an alternative, convenient method to functional studies in both animal and cell line models. Variety of RNAi vectors are now commercially available in the market.

Lentivectors are HIV-1 (Human Immunodeficiency Virus 1) derived plasmids, used to generate lentiviral particles (lentivirus) that can be transduced into virtually all kinds of mammalian cell types or organs, including stem cells, primary cells and non-dividing cells both ***in vivo*** and in **cell culture** system. Particles stably integrate into the transduced cells' genome for long term expression.

shRNA expression vectors with different markers:

Amsbio provides cloning kits for making shRNA lentiviral expression vectors with different selection markers (Cat#: **LTSH-GB, LTSH-GP, LTSH-RB, LTSH-RP, LTSH-Puro, LTSH-Bsd**).

Each kit contains a pre-cut, ready-to-use linear vector for ligation of shRNA duplex sequence. The linear vector was designed for cloning of double-strand DNA encoding a short hairpin RNA. Once transcribed, the shRNA is processed into short RNA *in vivo* for RNAi analysis. To make shRNA expression vector, two synthetic oligonucleotides are first annealed to form the DNA duplex which is then cloned into the ready-to-use, linear vector via T4 enzyme ligation. The transcription of shRNA is driven by tetracycline inducible human H1 promoter, a RNA polymerase III promoter.

The vector can be used for constitutive high level expression of shRNA without any induction. However, optionally, it can be used as **tetracycline inducible expression**. For inducible expression, the shRNA expression is repressed in the presence of TetR and induced by tetracycline. The expression of TetR can be achieved by using the Tet-repressor stable cell line (Cat# SC005) or pre-made **Tet-repressor lentiviral particles**, or co-transfection with the TetR expression vectors.

This lentiviral version of shNA vector allows generation of shRNA lentiviral particles that can be transduced into your desired cell lines. The shRNA stable expressing cells can then be selected by antibiotic or sorted via a fluorescent signal when applicable.

Each Kit provides enough materials for 10 cloning reactions, for generation of your own lentiviral shRNA expressing clones with the following advanced features.

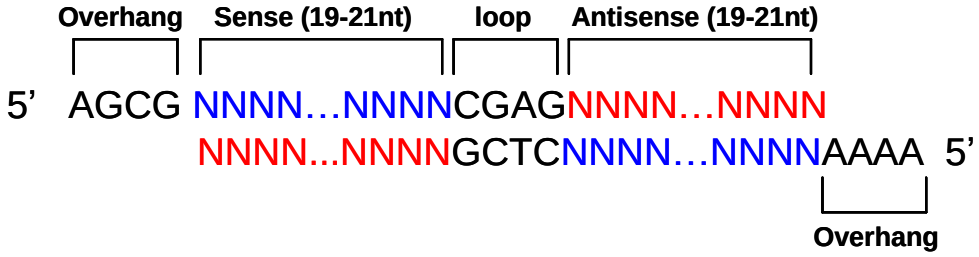
Key Features:

1. **Linearized vector** is ready for use, no need for the tedious bench work preparation of vector backbone;
2. **Precise directional cloning** of your DNA duplex encoded shRNA structure;
3. **Rapid, high efficient cloning** with low background (Room temperature for 30min, >90% positive rate);
4. **Internal fluorescent reference**: the vector encode a fluorescent protein (**GFP** or **RFP**), allowing real-time monitoring of the transfection or virus transduction efficiency;
5. **Long-term stable silencing effect**: the vector encodes an antibiotic marker or a **dual marker** (a fluorescent-antibiotic fusion marker) allowing generation of stable cell lines for long-term knockdown;
6. **Generated lentiviral shRNA particles can be transduced into your cells of interest (Note:** lentivector can be transfected into cells for gene expression knockdown. It can produce lentivirus to transduce the hard-transfected cells for long term knockdown study.)
7. **Optional inducible knockdown**: This lentivector (or its lentivirus) can be used for constitutive high expression of shRNA without needs for any induction. However, the vector's human H1 promoter is integrated with two Tet-repressor binding-sites (TRBS), allowing inducible expression of shRNA when the tetracycline repressor protein (TetR) exists in advance.
8. **Insert compatible:** the same annealed shRNA duplex can be readily cloned into all other linear shRNA lentivectors with different selection markers (Cat#: **LTSH-GB, LTSH-GP, LTSH-RB, LTSH-RP; LTSH-Puro; LTSH-Bsd**).

Protocols:

1. **Design single-stranded DNA oligonucleotide**:

   - **Design** two DNA oligonucleotides, a top strand and a bottom strand according to the following structure. The top strand has AGCG overhung at its 5'-end, followed by the selected target sequence (sense sequence) of 19-21 nucleotides, a CGAG loop (or use your own loop), and the reverse-complementary to the target sequence (antisense). The bottom strand has AAAA-overhung at its 5'-end and the rest is complementary to the top strand.



- **Loop length** has little or no effect on knockdown. Four nucleotides (CGAG) here have been tested as good, minimal length for effective RNAi knockdown. You may design your own loop sequence such as a restriction enzyme (RE) recognition sequence. However, most RE sequences are palindrome structures which form a continuous hairpin structure with your RNAi sequence, which may not be processed correctly into RNAi by Dicer *in vivo*.

- Two overhangs ensure the **directional cloning** of the annealed double stranded oligo into provided linear vector.

- The **transcription start site** is at the first nucleotide of the target sequence (sense) on the top strand. Native H1 RNA initiates at an A, so A is recommended to be the first base in sense target sequence.

- **shRNA target sequence (sense) selection**:

  There are some general guidelines for selecting the effective shRNA sequence. Many online tools or designers can help you select your shRNA sequence. But effective RNAi target sequence has to be empirically verified. To avoid the off-target effect, design a scrambled sequence (from selected shRNA sequence) or a universal Null sequence as a negative control for knockdown analysis.

  shRNA design tools:

  1. Promega's siRNA Target Designer;
  2. Clontech's RNAi Target Sequence Selector;
  3. Gene Link shRNA designer;
  4. Invitrogen's BLOCK-iT™ RNAi Designer;
  5. katahdin RNAi Central;
  6. WI siRNA selection program;

2. **Cloning of shRNA expression plasmids**:

- Anneal the designed two single-stranded DNA oligonucleotides:

  Set up the annealing reaction as follows:

100 µM Top strand oligo:	10 µl
100 µM Bottom strand oligo:	10 µl
10× oligo annealing buffer:	3 µl
DNase-free water:	7 µl
----------Total volume:	30 µl -------

  Incubate reaction mixture at 95°C for 5 minutes (can be done in PCR machine). Leave the mixture on the PCR machine to gradually cool down for **30 minutes**. Then put tubes on ice. Make 1:1000 dilution (add 1µl of annealed mixture in 99 µl cold-DNase free water, and then take 2 µl, add to 18 µl of 1x annealing solution on ice). Final diluted annealed duplex is ready for ligation. Save undiluted duplex at -

20˚C for long term storage. (**Note:** always put diluted, annealed duplex on ice to avoid double strand DNA melt.)

- Ligation reaction :

  Set up the ligation reaction as follows:

pEco-Lenti-H1-shRNA linear vector:	1 µl
Annealed duplex (1:1000 dilution):	1 µl
5x T4-ligase buffer:	2 µl
DNase free water:	5 µl
T4 ligase:	1 µl
----------Total volume:	10 µl -------

  Mix reaction well and incubate for 30 minutes at room temperature. (Note: incubation for longer time may generate more colonies.). Place reaction on ice and proceed with transformation.

  Set up a cloning **positive control reaction** by using **1 µl** of annealed Luc-shRNA duplex (provided, thaw on ice). The positive clone generated from control Luc-shRNA duplex is capable of silencing firefly luciferase gene (see "Example of knockdown" below in this manual).

- Transformation:

  1. Transfer 2µl of the ligation reaction into a vial of DH5α chemical competent *E. Coli* cells, gently mix. (**Note**: competent cells are not included in this kit).
  2. Place cells on ice for 5 minutes, then transfer cells into 42˚C water bath, incubate for 30 seconds without shaking. Immediately transfer cells to ice.
  3. Add 250µl of SOC medium; incubate at 37˚C for 1 hour with shaking.
  4. Spread all 250µl of trasnformed cells on a pre-warmed LB plate containing 100µg/ml **ampicillin**, and incubate overnight at 37˚C

  (**Note:** in general, you will get 30-100 colonies from your reactions and 0 to 5 colonies from the no-insert control reaction.)

3. **Confirm the positive clones**:

- Pick few colonies, grow in LB/ampicillin medium, miniprep plasmid DNAs, send for sequencing using the provided sequencing primer. (**Note:** Primer provided at ready to use concentration of 25ng/µl, simply use 1µl per reaction. Sequencing of stem hairpin structure may need special solution for best result.).
- Purified positive plasmid DNAs are ready for transfection into cells for knockdown analysis, or they can be used to produce lentiviral particles in packaging cell lines, and then the generated lentiviral particles can be used to transduce the cell line of your interest.

4. **Produce shRNA lentiviral particles (optional)**:

   Note: pEco-Lenti-shRNA vectors are fully compatible with most current lentiviral system on market. So you can use other vendor's lentivirus production system for virus production, such as ViraPower/Block-it (Invitrogen), MissionShRNA (Sigma), Lent-X (Clontech), GIPZ Lentiviral ShRNAmir (Open Biosystem), etc. But the following protocol is recommended for the highest virus titers using Amsbio lentiviral reagents.

   - **Cells**: grow packaging cells, 293T-LV (cat# **TLV-C**) in 24-well plate 2.5 x 10^5 cell/well), incubated in 5% CO_2 overnight.
   - **Transfection**: at the time of transfection, cells should be 90% confluent. Add 100µl of serum-free medium (or Opti-Mem, Invitrogen) into 1.5ml tube, then add 600ng of packaging mix (Cat# **HT-pack**) and 300ng of shRNA lentivector. Use your desired transfection protocols according to the transfection reagent manual. (For example: add 2µl of LF2K, incubated for 30min in room temperature).
   - Transfer all transfection mixture (~100ul) into a cell well in 24-well plate, incubated in 5% CO_2 overnight.
   - The next day, remove the medium and replace with complete culture medium.
   - Harvest virus supernatants at 48-72 hours after transfection.
   - Centrifuge virus particles at 3000rpm, for 15min at 4^{o}C to pellet cell debris. Filter through a sterile 0.45µm filter.
   - Store virus at -80˚C.

5. **Transduction of shRNA lentivirus and selection of the stable clones**:

   - **Cells**: plate the desired host cells at 10%-20% confluency, culture at 37˚C overnight;
     [**Note**: for inducible shRNA expression, a Tet-repressor stable cell line has to be used as host cells to repress the expression in advance and expression is induced later on by adding tetracycline. Amsbio provides a Tet-repressor expression cell line with Blasticidin selection (Cat# **SC005**)].
   - On 2nd day, thaw lentiviral stock, change medium with complete medium containing 6µg/ml polybrene; and add appropriate amount of lentiviral particles into culture to get a range of MOI from 1 to 10 as desired, incubate at 37˚C overnight;
   - At 24 hours after transduction, remove the medium containing virus and replace with complete medium, 37˚C overnight;
   - At 72 hours after transduction, remove the medium and replace with complete medium containing the appropriate amount of antibiotics to select for stably transduced cells. (Note: the amount of antibiotic added is dependent on the cell type. A kill curve has to be tested to use the right amount of antibiotics. In general, use 0.5-10µg/ml of blasticidin and 10-100 µg/ml of puromycin).
   - Change medium containing puromycin every 3-4 days;

- At the time when the mock treated well has no living cells, trypsinize the antibiotic-resistant colonies and make a series of dilution, seed into each well in 24-well plate, continue to grow cells,
- Inspect the cells under fluorescent microscope, select the wells that show GFP signal from all cells, grow cells in larger amounts;
- Collect cells, and freeze down cells in cryogenic vial, as stable shRNA expression cell lines;

Validation of shRNA knockdown:

In general, most RNAi designs can obtain greater than 50% success rate with greater than 75% knockdown levels. However, there is no "holy-grail" for an ultimate RNAi design. Effective RNAi sequence has to be empirically validated. To validate effective shRNAs, different approaches are used to measure the mRNA levels or its protein products, such as using Q-PCR or western blot. Alternatively, a reporter assay can be applied to screen shRNAs.

One main concern for RNAi knockdown is the so called "off target effect". We designed a negative shRNA sequence as the universal negative control. It was designed against entire human and mouse transcripts with the minimal sequence humology to any human or mouse ORF sequence to minimize the non-specific knockdown. The **Negative-control shRNA lentiviral particles** are provided as catalog products. Or you can design and clone your own negative control shRNA using this kit.

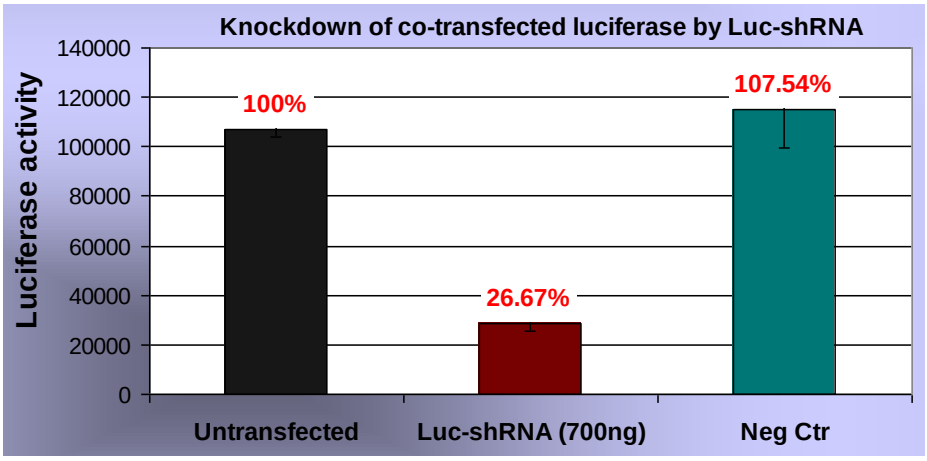
For you reference, please review the following expression knockdown results using Amsbio's shRNA vector:

- Examples for knockdown using pEco-H1-shRNA-(GFP-Bsd) vectors:

  Example A: Luc-shRNA: (measure the luciferase activity by luciferase assay kit)

  Luc-shRNA top strand:

  5'- AGCGatgaaacgatatgggctgaatacCGAGgtattcagcccatatcgtttca

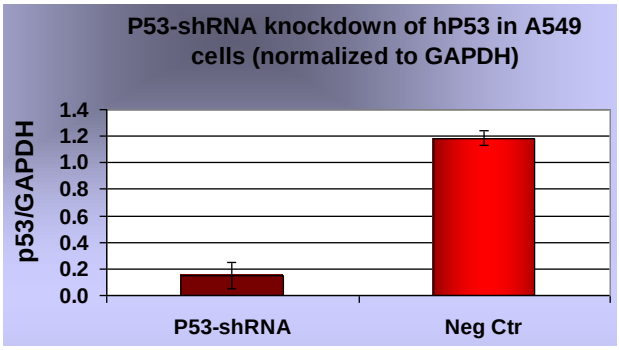


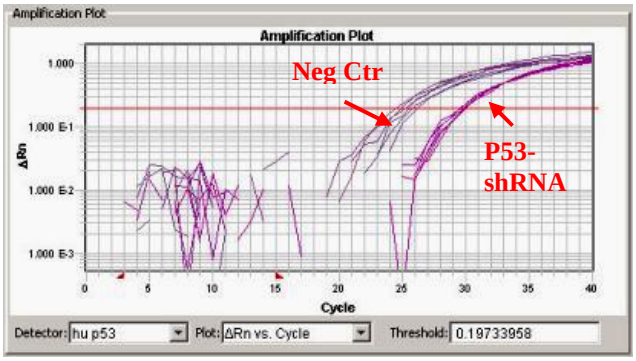
Knockdown of co-transfected luciferase expression in 293-HEK cells by pEco-H1-luc-shRNA-(GFP-Bsd) plasmid.
Luc-shRNA duplex was cloned into pEco-H1-shRNA-(GFP-Bsd) vector, then co-transfected with pcDNA3.1-luciferase (firefly) plasmid (100ng) and Luc-sh-RNA plasmid (700ng) into 293HEK cells in 24-well plate. Cells were harvested at 3 days after transfection. Luciferase activity was measured from cell lysate (10ul/ea) using luciferase reporter assay kit on LMax microplate luminometer. Null-shRNA plasmid serves as the negative control plasmid here.

Example B: P53-shRNA: (Measurement of mRNA level by real-time qPCR)

P53-shRNA top strand:

5'- AGCGccactacaactacatgtgtaaCGAGttacacatgtagttgtagtgg





Knockdown of endogenous human P53 in A549 cells by pEco-H1-p53-shRNA-(GFP-Bsd) plasmid. P53-shRNA duplex was cloned into pEco-H1-shRNA-(GFP-Bsd) vector, and transfected into A549 cells, grown in medium containing 10µg/ml blasticidin. Cells were harvested at 3 days after transfection. P53 levels were detected from extracted total RNA by real-time Q-PCR assay. Data were normalized to internal level of GAPDH. Null-shRNA plasmid serves as negative control.

Conclusion: RNAi gene silencing can be effectively carried out via pEco-H1-shRNA-(GFP-Bsd) vectors.

Trouble shooting:

Problems	Solution
Few or no colonies	1. Make freshly annealed duplex, and dilute for ligation reaction; 2. Extend ligation time, or leave it at 4°C overnight; 3. Use more duplex: add 5µl of diluted duplex in ligation reaction; 4. Use different competent cells;

Related Products:

Cat#	Product Name	Amount	Application
HT-Pack	Lentiviral packaging plasmids	600ng/µl x 100µl	Packaging for lentivirus production
TLV-C	293TLV lentiviral packaging cells	One vial (> 2 x 10^6 cells)	lentivirus production cells

Lentiviral shRNA cloning service:

Amsbio provides cost effective shRNA cloning services. Simply tell us the target you want to knockdown, we will design the shRNA for your target (or you provide the RNAi target sequence), and we clone shRNA sequences into our shRNA expression vectors with the selected marker. Sequencing verified shRNA plasmids and packaged lentiviral shRNA particles will be delivered to you. Our service has the fast turnaround time and lowest costs. Please contact us for quote.

References:

1. Lee, R. C., et al, The C-elegans Heterochronic Gene lin-4 Encodes small RNAs with antisense complementarily to lin-14. Cell, 75(843-854), 1993.
2. Hannon, G.J., RNA interference. Nature, 418(6894): p. 244-51, 2002.
3. Bosher, M., et al, RNA interference, Nature Cell Biol. 2 E31-E36, 2000.
4. Meister, G. and T. Tuschl, Mechanisms of gene silencing by double-stranded RNA. Nature, 2004. 431(7006): p. 343-9.
5. Paddison, P.J., A.A. Caudy, and G.J. Hannon, Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci U S A, 2002. 99(3): p. 1443-8.